Antioxidative Potential of Fluvastatin via the Inhibition of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase Activity

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We previously reported that fluvastatin, a potent 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is a strong lipid lowering drug, exerting an anti-atherosclerotic effect at doses insufficient to lower serum lipids in cholesterol-fed rabbits. The evidence demonstrated that the superoxide anions from nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) plays a critical role in several steps in the development of atherosclerosis. This study was designed to determine the effects of HMG-CoA reductase inhibitors on the production of the superoxide anions of NADPH oxidase in isolated rat peritoneal neutrophils. Fluvastatin (1–10 μM) decreased phorbol 12-myristate 13-acetate (PMA, 10 nM)-dependent reactive oxygen species (ROS) generation in a concentration-dependent manner. It also (10 μM) decreased PMA-dependent O2 consumption of the rat neutrophils. These effects were reversed by the addition of mevalonate, a metabolite in the HMG-CoA reductase pathway. Treatment with pravastatin did not show any significant changes. Fluvastatin (10 μM) decreased ROS, such as hydroxyl radicals and superoxide anions generated by the Fenton reaction, and by the xanthine–xanthine oxidase system. Rats were treated with either fluvastatin (5 mg/kg per day, p.o.) or pravastatin (5 mg/kg per day, p.o.) for 1 week. Treatment with fluvastatin decreased the PMA-dependent ROS generation. The fluvastatin-induced effect on the PMA-dependent ROS generation was reversed by the combined administration with 40 mg/kg mevalonate per day. The antioxidant effect of fluvastatin was thought to have caused not only the scavenging action of the radicals but also to have inhibited ROS generation by inhibiting the NADPH oxidase activity. This antioxidant potential of fluvastatin via the inhibition of NADPH oxidase activity may be profitable in preventing atherosclerosis.

Key words fluvastatin; nicotinamide adenine dinucleotide phosphate oxidase; antioxidant; 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor

Oxidative stress plays a critical role in the pathogenesis of atherosclerosis.1–3 Elevated oxidative stress and superoxide anion generation could promote the conversion of low density lipoprotein (LDL) to atherogenic oxidized LDL (ox-LDL), contributing to atherosclerosis. There is increasing evidence that the oxidative modification of LDL contributes to the formation of foam cells in the artery wall.4 Concerning the modification of LDL, neutrophils rapidly take up ox-LDL and generate superoxide anions, which may cause superoxide mediated lipid peroxidation in vivo.4 The superoxide anions of human monocytes participate in both the oxidation of LDL and its conversion to a cytotoxin.5

We had reported that fluvastatin, a strong inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the late limiting enzyme of cholesterol biosynthesis, suppresses atherosclerotic progression, mediated through its inhibitory effect on endothelial dysfunction, lipid peroxidation, and macrophage deposition, unrelated to its strong hypcholesterolemic action.6 Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is an important and major source of superoxide anions in neutrophils.6

Thus, the aim of this study was to determine the direct effect of fluvastatin on the NADPH oxidase activity in neutrophils using phorbol 12-myristate 13-acetate (PMA) as a stimulant to clarify the strong anti-atherosclerotic effect without serum lipid reduction. PMA activates protein kinase C (PKC) directly, and PKC activity is primarily cytosolic in unstimulated neutrophils, but becomes firmly associated with the membrane fraction after PMA treatment.7 The effects of fluvastatin on NADPH oxidase activity were also compared with those of another HMG-CoA reductase inhibitor, pravastatin, which is known as a highly hepatocyte-selective agent in inhibiting cholesterol synthesis.8

MATERIALS AND METHODS

Chemicals PMA was obtained from Sigma (St. Louis, MO, U.S.A.). L-012 was donated by Wako Pure Chemical Co. (Osaka, Japan). The drugs used were fluvastatin, supplied by Sandoz Research Institute (E. Hanover, NJ, U.S.A.), and pravastatin obtained from Sankyo (extracted from Mevalonat®; Tokyo, Japan). Mevalonate was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Male Wistar rats, 6 weeks old, were supplied from SLC (Shizuoka, Japan). The spin-trapping agent, 5,5-dimethylpyroline-N-oxide (DMPO), was obtained from Sigma (St. Louis, MO, U.S.A.). Ammonium Iron (II) Sulfate Hexahydrate was obtained from Wako Pure Chemical Co. (Osaka, Japan). Superoxide dismutase (SOD) was obtained from Chiron Corporation (CA, U.S.A.).

Preparation of Neutrophils Neutrophils were obtained from 6-week-old male Wistar rats 16 h after an intraperitoneal injection of 2% casein solution at a dosage of 1/10 the body weight, as described by Morimoto et al.11 After 16 h, migrated neutrophils were collected by an intraperitoneal injection of 50 ml calcium-free 0.9% NaCl solution containing 10 mM phosphate buffer (pH 7.4), 6 mM KCl, and 1 mM MgCl2 (Kreb’s–Ringer phosphate buffer solution, KRP). The neutrophils were washed 3 times with KRP solution by cen-
trifugation (1500 rpm, 5 min, 4 ℃). The pellet in the bottom of the tube was collected and then resuspended in KRP (1×10^6 cells/ml) and kept on ice until used for the experiments.

**Assay for ROS Generation** The neutrophils (10^6 cells/ml) were incubated in 0.5 ml of KRP in the presence of 400 μM L-012 and fluvastatin or pravastatin of each concentration. After incubation for 5 min at 37 ℃, the reaction was started by the addition of 10 nM PMA. During the incubation, the chemiluminescence (CHL) intensity was recorded continuously for 20—30 min using a Luminescence Reader BLR-201 (Aloka, Tokyo, Japan). Cell viability was assessed by trypan blue exclusion after 30 min incubation with the test compounds, and the compounds did not show cytotoxicity under these conditions. One hundred μM mevalonate was dissolved in ethanol and added to the reaction mixture at a final concentration of 0.1% or less, and mevalonate and ethanol did not show any effect on reactive oxygen species (ROS) generation. Values were expressed as a percent change from the control mean values.

**Hydroxyl Radical Analysis** The final concentrations of the mixtures were set at 200 μM H2O2, 100 μM FeSO4·(NH4)2SO4·6H2O, 1 mM DMPO, 50 mM potassium phosphate buffer (pH 7.0), and fluvastatin or pravastatin of each concentration. The signal intensities were evaluated as the peak height of the second signal of the quartet of DMPO-OH spin adducts. ESR spectra were measured with a JEOL JES-TE200 ESR spectrometer (Tokyo, Japan). Typical ESR settings were, field, 3350 G; frequency, 9.42 GHz; and modulation, 100 kHz×1G. Values are expressed as a percent change from the control mean values.

**Superoxide and O2 Consumption Analysis** Superoxide anion was generated by the xanthine–xanthine oxidase system. The concentrations of the mixtures were set at 0.05 mM xanthine, 50 mM phosphate buffer (pH 7.8), 1 μM cytochrome c, 0.01 μM xanthine oxidase, and fluvastatin or pravastatin of each concentration. The direct superoxide anion scavenging activity of fluvastatin or pravastatin was measured by the cytochrome c method at 550 nm using a Shimadzu UV-3100 PC scanning spectrophotometer. Values are expressed as a percent change from the slope before the addition of each drug as a control.

O2 consumption by neutrophils was measured with an oxygen electrode using a Lank Brothers LTD. oxygen monitor connected to a Pantos uniconder, U-228 recorder. Assays were conducted at 37 ℃ with 4×10^6 cells/ml. The reaction was started by the injection of 10 nM PMA into the 2 ml chamber. Values were expressed as a percent change from the slope of O2 consumption before the addition of each drug, as a control.

**Animal Treatment** Male Wistar rats (6 weeks old) were obtained from SLC (Shizuoka, Japan). The animal room was maintained at 24±2 ℃, with 55±10% relative humidity, and a 12 h light/dark cycle. The rats were given water and commercial laboratory chow (MF; Oriental Yeast Co., Japan) ad libitum for at least one week before use. Rats were given oral fluvastatin or pravastatin at a dose of 5 mg/kg once a day in the evening (17:00—18:00) for one week. Mevalonate was given orally with fluvastatin at a daily dose of 40 mg/kg. The control group was given distilled water. Values were expressed as a percent change from the control mean values.

**Data and Statistical Analysis** The data were expressed as the mean±S.E. Statistical analyses of the data were performed using one-way analysis of variance, followed by Dunnett multiple comparison test. Statistical significance was accepted at p<0.05.

**RESULTS**

To determine the effect of fluvastatin on the NADPH oxidase activity in neutrophils, the effects of fluvastatin on ROS generation and O2 consumption in rat neutrophils were examined.

By adding 1 and 10 μM fluvastatin to the neutrophil suspensions, ROS generation was significantly decreased in a dose dependent manner, and this effect was reversed by the addition of 100 μM mevalonate. In contrast with fluvastatin, ROS generation was not significantly decreased by treatment with the same concentration of pravastatin, the other HMG-CoA reductase inhibitor (Fig. 1).

O2 consumption of rat neutrophils was significantly decreased by treatment with 10 μM fluvastatin, and this effect was reversed by the addition of 100 μM mevalonate. On the other hand, pravastatin did not show any significant effect on O2 consumption up to 10 μM (Fig. 2).

The direct hydroxyl radical scavenging effect was determined by ESR studies. In order to evaluate the potency of the scavenging effect of fluvastatin on hydroxyl radicals, we compared the effect with pravastatin. Ten μM fluvastatin showed a significant hydroxyl radical scavenging effect. In contrast, pravastatin did not show any significant effect on the quantity of hydroxy radicals up to 10 μM (Figs. 3, 4).

The direct superoxide anion scavenging activity of fluvastatin was measured by the cytochrome c method. SOD effectively decreased the increasing rate of the absorbance, suggesting that the generation of superoxide anion actually occurred in the mixture. Ten μM fluvastatin showed significant scavenging activity of superoxide anions. In contrast, pravastatin did not show any significant effect on the quantity of superoxide anions (Fig. 5).

To confirm whether these in vitro effects actually appear in vivo, fluvastatin and pravastatin were administered orally to male Wistar rats (6 weeks old), and the effects of these drugs on ROS generation were evaluated.

![Fig. 1. Effects of HMG-CoA Reductase Inhibitors on PMA-Dependent ROS Generation in Isolated Rat Neutrophils](image-url)
the rats once a day for 1 week. Treatment with fluvastatin (5 mg/kg per day, p.o.) for 1 week decreased the PMA-dependent ROS generation. The fluvastatin induced effect on the PMA-dependent ROS generation was reversed by the combined administration with 40 mg/kg mevalonate per day. Pravastatin did not show any significant effect (Fig. 6).

DISCUSSION

In the present study, fluvastatin significantly decreased the PMA-dependent ROS generation. It was thought that this decreasing effect of ROS generation with fluvastatin was, at least in part, attributed to a direct inhibitory action to neutrophil NADPH oxidase, because the O$_2$ consumption of neutrophils was significantly decreased by treatment with fluvastatin. This inhibitory effect of fluvastatin on ROS generation and O$_2$ consumption was reversed by the addition of mevalonate, a metabolite of the HMG-CoA reductase pathway. This phenomenon indicated that fluvastatin inhibits ROS generation via the inhibition of the cholesterol synthesis pathway.

Fig. 2. Effects of HMG-CoA Reductase Inhibitors on O$_2$ Consumption in Isolated Rat Neutrophils
Fluvastatin (FV), pravastatin (PV), and mevalonate (MEV), were added 3.5—4 min after the PMA addition. Values are mean±S.E. (n=6). Values are expressed as a percent change from the basal slope of O$_2$ consumption before the addition of each drug, as a control. The basal slope before the addition of each drug was –4.30±0.15 (mm/min). *p<0.05, significantly different from the control group (C, Dunnett multiple comparison test).

Fig. 3. Typical Electron Spin Resonance (ESR) Spectra of DMPO-OH Generated by the Fenton Reaction
The ESR spectra after the reaction of 1 mAo DMPO with 200 μm H$_2$O$_2$ and 100 μm FeSO$_4$/NH$_4$SO$_4$·6H$_2$O in the absence (control), and in the presence of 10 μm fluvastatin (FV) or 10 μm pravastatin (PV).

Fig. 4. Hydroxyl Radical Scavenging Effects of HMG-CoA Reductase Inhibitors
The concentration of the DMPO spin adducts of the hydroxyl radicals (DMPO-OH) formed was recorded after the addition of DMPO. FV: Fluvastatin, PV: Pravastatin, C: Control. Values were mean±S.E. (n=4). *p<0.05, significantly different from the control group (Dunnett multiple comparison test).

Fig. 5. Superoxide Anion Scavenging Effects of HMG-CoA Reductase Inhibitors
Superoxide anions were generated by the xanthine–xanthine oxidase system. The direct superoxide radical scavenging activity of HMG-CoA reductase inhibitors was measured by the cytochrome c method. FV: Fluvastatin, PV: Pravastatin, C: Control, SOD: 33 μg/ml. Values are mean±S.E. (n=4). Values are expressed as a percent change from the slope before the addition of each drug, as a control. The basal slope of the control group was 0.027±0.002 (Absorbance at 550 nm/min). *p<0.05, significantly different from the control group (Dunnett multiple comparison test).

Fig. 6. In Vivo Effects of HMG-CoA Reductase Inhibitors on PMA-Dependent ROS Generation
Fluvastatin (FV, 5 mg/kg, p.o.) and pravastatin (PV, 5 mg/kg, p.o.) were administered once a day for 1 week. Mevalonate (MEV) was given orally with fluvastatin for 1 week at a daily dose of 40 mg/kg, C: Control. Values are mean±S.E. (n=6). The basal value of the control group was 52.1±7.1 (cpm×10$^9$). *p<0.05, significantly different from the control group (Dunnett multiple comparison test).
pathway. The neutrophil oxidase consists of 5 major subunits: a plasma membrane spanning cytochrome b558 composed of a large subunit gp91phox and a smaller subunit p22phox, and 3 cytosolic components p40phox, p47phox and p67phox.13—15) The low molecular weight G protein rac2 participates in the assembly of the active complex and NADPH oxidase is activated by isoprenylation of the rac2 protein, which results in a production of superoxide anions.16) HMG-CoA reductase generates mevalonate from HMG-CoA, and mevalonate is further metabolized into a series of isoprenoids. Accordingly, fluvastatin may suppress the isoprenylation of the rac2 protein, and may finally suppress the production of superoxide. The fact that the inhibitory effect of fluvastatin on superoxide generation was reversed by the addition of mevalonate is also further evidence to the possibility. Inoue et al. reported that the mRNA levels of p22phox, and the protein levels of p47phox of NADPH oxidase, were decreased by treatment with fluvastatin, and that this effect was reversed by the addition of mevalonate.17) To understand the detailed mechanism by which fluvastatin inhibits neutrophil superoxide anion production, additional studies such as an analysis of the translocation of cytosolic subunits (p40phox, p47phox and p67phox) to the membrane, and their assembly with the gp91phox and p22phox subunits, is required.

Fluvastatin reduced active oxygen species such as hydroxyl radicals and superoxide anions, generated by the Fenton reaction and by the xanthine–xanthine oxidase system, respectively. There are several reports that fluvastatin suppresses lipid peroxidation by scavenging active oxygen species such as hydroxyl radicals and the superoxide anions of rat liver microsomes in vivo and in vitro.18,19) Pravastatin did not show a chemical scavenging action, such as hydroxyl radicals and superoxide anions. It is assumed that these phenomena originate in its chemical structure.

Thus, it was confirmed that fluvastatin decreased PMA-dependent ROS generation in vitro, and we further examined the in vivo effects of fluvastatin on PMA-dependent ROS generation to confirm whether the observed in vitro effects actually occur in vivo. Treatment with fluvastatin (5 mg/kg per day, p.o.) for 1 week also decreased PMA-dependent ROS generation in vivo, and this suppressive effect of fluvastatin on ROS generation was also reversed by the combined administration with mevalonate. Pravastatin with the same concentration and dose as fluvastatin did not show any significant effect on ROS generation in vitro or in vivo. The most likely reason for the difference between these HMG-CoA reductase inhibitors is that greater doses of pravastatin are required to produce these effects in this species of animals and cells. However, pravastatin showed a similar range of lipid lowering effects in humans at 0.3—0.7 mg/kg,20) 20—40 mg/d for pravastatin versus 20—60 mg/d for fluvastatin.21) Pravastatin is known as a highly hepatocyte-selective agent in inhibiting cholesterol synthesis.22) Pravastatin is actively transported into hepatocytes via a carrier mediated system. Conversely, in non hepatic cells, the uptake of pravastatin is minimal over a wide concentration range because of its greater hydrophilicity.23) It was reported that the IC₅₀ values of the cholesterol synthesis with fluvastatin and pravastatin on aortic smooth muscle cells were 0.15 μM and 195.0 μM, respectively.24) It may not actually inhibit neutrophil cholesterol synthesis, and it is possible that pravastatin cannot suppress the isoprenylation of the rac2 protein of the neutrophils. In addition, different characteristics between pravastatin and fluvastatin are also found, with respect to a lack of the inhibitory effect on the thickening of the intima after balloon catheterization.23) Thus, although the dose factor cannot be excluded, pravastatin might be less permeable to neutrophils than fluvastatin, as shown by the liver specificity of the former drug action based on its hepatocytes specific carrier mediated system and greater hydrophilicity.25) In this in vivo model, it is considered that fluvastatin did not alter serum lipid concentration. Tsujita et al. reported that pravastatin did not reduce serum lipids in rats and mice.24) Furthermore, Kimura et al. reported that fluvastatin also did not reduce serum lipids at a dose of 6 mg/kg in rats.25)

Recently, it was demonstrated that the oxidative stress by NADPH oxidase in endothelial cells (EC) also plays a role in the early steps of atherosclerosis.26) Meyer et al. confirmed protein expression of NADPH oxidase subunits, such as gp 91phox, p22phox, p47phox, and p67phox in EC, and that apocynin, a specific leukocyte NADPH oxidase inhibitor, inhibits the translocation of p47phox to the membrane of stimulated EC. These findings also support the presence of a functionally active leukocyte type NADPH oxidase in EC.27) From these facts, it is likely that fluvastatin inhibits the progression of atherosclerosis via the inhibition of NADPH oxidase in not only neutrophils but also in EC. Rueckschoess et al. reported that oxidized LDL induces proatherosclerotic NADPH oxidase expression and superoxide anion formation in human endothelial cells, and an antioxidative potential of HMG-CoA reductase inhibition via the reduction of vascular NADPH oxidase expression.28) Furthermore, Inoue et al. reported that after the 16-week lipid-lowering therapy, the anti-ox-LDL titer significantly decreased in the fluvastatin group but did not change in the pravastatin group.29)

Several kinds of molecules such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, are known as reactive oxygen species which neutrophils produce, beside superoxide anions. At first, superoxide anions are produced, and then other reactive oxygen species are produced from superoxide anions by the chemical reaction. Accordingly, NADPH oxidase takes part directly only in the production of superoxide anions.30) Therefore, it was thought that the direct NADPH oxidase inhibition of fluvastatin is important.

NADPH oxidase is present in neutrophils and EC, and it seems to be a source of superoxide anion production in animal models of vascular disease, and in human atherosclerosis.31) Therefore, these effects of fluvastatin may be beneficial in preventing vascular complications in hyperlipidemia, in addition to its strong hypocholesterolemic action.

REFERENCES